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Unusual non-enzymatic flavin catalysis enhances understanding of flavoenzymes

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ABSTRACT

Flavin cofactors are central to many biochemical transformations and are typically tightly bound as part of a catalytically active flavoenzyme. This work indicates that naturally occurring flavins can act as stand-alone catalysts to promote the oxidation of biosynthetically inspired heterocycles in aqueous buffers. Flavin activity was compared with that of oxidases important in non-ribosomal peptide synthesis, providing a rare direct comparison between the catalytic efficacy of flavins alone and in the context of a full flavoenzyme. This study suggests that such oxidases are likely to possess an active site base, as oxidase activity was greater than that of flavins alone, particularly for less acidic substrates. These findings offer perspective on the development of robust and catalytically effective, designed miniature flavoenzymes.

1. Introduction

Flavins are redox cofactors central to numerous metabolic processes [1]. As such, they are nearly always tightly associated with flavoenzymes, and this complex together is responsible for the catalysis of a wide range of biochemical transformations [2]. While flavoenzymes are well-known in primary metabolic pathways, they also play an important role in secondary metabolism [3]. Flavin-binding oxidases are located in biosynthetic pathways yielding nitrogen-containing heterocycles such as thiazoles and oxazoles. These heteroaromatic rings are formed from post-translational modification of serine, threonine and cysteine in the ribosomal production of peptides like microcin B17 [4] and are also found in non-ribosomal peptides formed by non-ribosomal peptide synthetases (NRPS) [5]. Oxidases in such biosynthetic pathways facilitate the oxidation of thiazoline and oxazoline rings to the more stable, aromatic thiazole and oxazole rings, often necessary for the bioactivity of the natural product. Because these nitrogen-containing heteroaromatic rings are of interest, particularly from a medicinal chemistry perspective, their formation has been investigated.

The NRPS oxidases important for the biosynthesis of the anti-cancer agents epothilone and bleomycin have been characterized, with catalytic activity for a series of thiazoline and oxazoline substrate mimics measured [6]. These oxidases were found to bind flavin mononucleotide (FMN) non-covalently but so tightly that denaturation is necessary to separate the two. Flavin cofactors are not usually exchanged after catalytic turnover and tend to be regenerated while enzyme-bound, requiring molecular oxygen or other oxidants. Because flavins can be simply regenerated by molecular oxygen, and are linked to the formation of bioactive small molecules, they are intriguing targets for stand-alone bioinspired catalysts.

Some synthetic flavin analogs have been evaluated, suggesting their utility as effective and environmentally mild oxidants for synthetic transformations [7]. Hydroperoxyflavins recently have been shown to promote the formation of heteroaromatic pyridines and benzothiazoles at ambient temperature in methanol and in the presence of molecular oxygen [8]. This report joins other work describing synthetic flavins capable of catalyzing the oxidation of amines, sulfides and aldehydes [9–11]. These synthetic oxidation reactions take advantage of the redox potential of flavins as "green" catalysts while removing the enzyme portion of flavoenzymes which can be more susceptible to denaturation under varied solvent conditions.

In this work, the potential of naturally occurring flavins as stand-alone catalysts mediating the oxidation of biosynthetically inspired small molecules under physiologically relevant conditions

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was probed. FMN, flavin adenine dinucleotide (FAD), and riboflavin all catalyze the formation of heteroaromatic rings in thioester-linked small molecules known to be substrates for NRPS oxidases. As these reactions occur in aqueous buffers mirroring the reaction conditions needed for oxidase activity, this study allows a rare direct comparison between the catalytic efficacy of flavins alone and in the context of the full flavoenzyme. This analysis provides mechanistic insights on the catalytic role of the oxidase enzyme beyond the bound flavin cofactor and offers perspective on the development of robust and catalytically effective, designed miniature flavoenzymes.

2. Materials and methods

2.1. Reagents and enzymes

FMN, FAD and riboflavin were purchased from Sigma–Aldrich. Reagents for the synthesis of substrates and product standards were also purchased from Sigma–Aldrich. Other reagents were purchased from Fisher Scientific unless otherwise specified. Overexpression and purification to yield EpoB-Ox were performed as described [6]. To determine flavin-bound enzyme concentration, a sample of purified EpoB-Ox was heat denatured for 2 min at 90 °C, and the supernatant was subjected to UV–vis analysis. Concentration was calculated based on the known extinction coefficient for FMN, 12 200 cm^{-1} M^{-1} at 450 nm [12].

2.2. Chemical synthesis of substrates and standards

Phenylthiazoline carboxylic acid, phenylthiazole carboxylic acid, phenylthiazolinyl-*S*-NAC, phenylthiazolyl-*S*-NAC, phenyloxazolinyl-*S*-NAC, phenyloxazolyl-*S*-NAC, methylthiazolinyl-*S*-NAC and methylthiazolyl-*S*-NAC were synthesized as described [6,13,14].

Hydrocinnamyl-*S*-NAC was prepared by combining hydrocinnamic acid (73.0 mg, 486 μmol), *N*-acetylcysteamine (NAC; 78 μL, 730 μmol), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC; 140 mg, 730 μmol) and triethylamine (TEA; 102 μL, 732 μmol) in dichloromethane. After stirring at 25 °C for 18 h, the reaction was evaporated to dryness and resuspended in ethyl acetate. This mixture was washed once each with 10% hydrochloric acid, saturated sodium bicarbonate and brine. The remaining organic layer was evaporated to dryness to yield an oil (82%) that was used without further purification. ^{1}H NMR (500 MHz, $CDCl_3$): 7.26 (t, 2H), 7.18 (m, 3H), 6.00 (s, 1H), 3.37 (q, 2H), 2.98 (m, 4H), 2.87 (t, 2H), 1.91 (s, 3H).

Cinnamyl-*S*-NAC was prepared by combining cinnamic acid (72.0 mg, 480 μmol), NAC (78 μL, 730 μmol), EDC (140 mg, 730 μmol) and TEA (102 μL, 732 μmol) in dichloromethane. After stirring at 25 °C for 18 h, the reaction was evaporated to dryness and resuspended in ethyl acetate. This mixture was washed once each with 10% hydrochloric acid, saturated sodium bicarbonate and brine. The remaining organic layer was evaporated to dryness to yield an oil (40%) that was used without further purification. ^{1}H NMR (500 MHz, $CDCl_3$): 7.59 (d, 1H), 7.51 (m, 2H), 7.35 (m, 3H), 6.69 (d, 1H), 6.31 (s, 1H), 3.46 (q, 2H), 3.13 (t, 2H), 1.95 (s, 3H).

2.3. FMN and oxidase activity assays

Reactions containing FMN or EpoB-Ox were carried out in 75 mM sodium phosphate, pH 6.0, 7.0, or 8.0, or in 75 mM Tris, pH 8.8. Reaction mixtures also included 8% THF for substrate solubility. Assays comparing FMN and EpoB-Ox activity at varied pH were performed with 2 mM phenylthiazoline-*S*-NAC and 10 μM catalyst. Reactions, performed in triplicate, were quenched after 1 h upon HPLC injection. Phenylthiazolyl-*S*-NAC product was detected by analytical HPLC (Agilent 1100 Series) using a linear gradient of acetonitrile in water from 40% to 55% over 8 min, resolved by a 4.6 × 150 mm Eclipse XDB-C18 column (Agilent). In all cases, product formation was quantified by integrating peak area and comparing it with a standard curve prepared with synthetic phenylthiazolyl-*S*-NAC. Reported product formation in each case represents values corrected for the small amount of product formed when no catalyst was present. Product identity was confirmed by collecting the putative product peak and analyzing its mass by LC-MS (Thermo Scientific Surveyor LC system and LTQ XL mass spectrometer with electrospray ionization).

Kinetic parameters for reactions with FMN were determined with 3 μM FMN, 75 mM sodium phosphate, pH 7.0, and a range of phenylthiazolinyl-*S*-NAC concentrations in a total volume of 50 μL. Reactions were quenched after 4 h, a time point within the linear range for this reaction, by HPLC injection. This experiment was carried out in triplicate, data were fit with the Michaelis–Menten equation, and the reported error indicates the standard deviation among the three trials.

2.4. Flavin activity comparison

Assays to compare the initial rate of product formation with FMN, FAD and riboflavin were carried out in 75 mM sodium phosphate, pH 7.0 with 3 μM flavin and 3.1 mM phenylthiazolinyl-*S*-NAC. Reactions were quenched at a series of time points by HPLC injection. A linear fit was used to determine initial velocity under these conditions for each flavin. Kinetic parameters for FAD were determined as described above for FMN.

3. Results and discussion

While previous work with flavin-binding NRPS oxidases determined their catalytic efficacy in the biosynthesis of heteroaromatic rings [6], here, the possibility of flavin cofactors alone as catalysts in similar reactions was evaluated. FMN, the flavin bound by NRPS oxidases, was incubated with phenylthiazolinyl-*S*-NAC in sodium phosphate buffer, pH 8, at ambient temperature. After 1 h, a new peak was detected by HPLC that coeluted with chemically synthesized phenylthiazolyl-*S*-NAC (Fig. 1). The identity of the new peak was further confirmed by LC–MS ($[M+H]^+$ observed, 307.2; calculated 307.1). Significantly less phenylthiazolyl-*S*-NAC product was observed in control reactions containing no FMN. This result indicates that FMN alone can act as an oxidation catalyst in the preparation of heteroaromatic small molecules under aqueous conditions.

The catalytic activity of FMN alone was compared with that of the known NRPS oxidase EpoB-Ox, a flavoenzyme required for the biosynthesis of the thiazole ring within the anticancer agent epothilone [6]. Inspection of the catalytic activity of the two at 0.5 mol% revealed that both promote product formation at a range of pH values, as measured after 1 h at ambient temperature (Fig. 2A). Reactions with either catalyst yielded more product as buffer pH increased, supporting an oxidation mechanism that requires base-mediated deprotonation of the substrate in addition to hydride transfer to the flavin (Fig. 2B). Perhaps not surprisingly, this series of assays suggests that EpoB-Ox is the more robust catalyst, with a proportionately greater impact at lower pH than FMN alone. In order to further compare the activity of FMN and EpoB-Ox, kinetic parameters for FMN alone were determined in assays at pH 7 (Fig. 2C), matching the conditions used for the Michaelis–Menten analysis previously completed with EpoB-Ox [6]. While the K_m in each case was similar (1.1 ± 0.1 mM for FMN; 2.1 ± 0.4 mM for EpoB-Ox), the rate constant measured for